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Mycobacterial adenosine kinase is not a typical adenosine kinase

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ABSTRACT

Adenosine kinase (AK) is only found in eukaryotes. Recently, a *Mycobacterium tuberculosis* (MTub) protein exhibiting greater sequence similarity to ribokinases (RK) was identified as AK. We have expressed AKs from MTub, human and Chinese hamster (CH) cells in *Escherichia coli* and also AK from human and MTub in AK-deficient CH cells. While both *E. coli* and CH cells expressing mammalian AKs efficiently metabolized various adenosine analogs, those expressing MTub-AK were completely inactive. The AK activity of the MTub protein was very low (50-fold lower than *E. coli* RK) and it was not stimulated by phosphate or inhibited by several AK inhibitors. These results raise questions over MTub protein's true function and whether it functions as AK in cells.

1. Introduction

Adenosine kinase (AK) catalyzes the phosphorylation of adenosine (Ado) to adenosine monophosphate (AMP) using the γ-phosphate of the co-substrate Mg/ATP^{-2}. AK belongs to the phosphofructokinase B type (PfkB) family of sugar kinases, which includes many proteins such as ribokinase (RK), inosine-guanosine kinase, fructokinase, and 1-phosphofructokinase [1–3]. Recent studies on AK have focused on designing inhibitors, particularly of the human and parasitic protozoan forms. In humans, it has been proposed that inhibitors of AK could produce various pharmacological effects by increasing intravascular Ado concentrations and act as anti-inflammatory agents [3,4]. In parasitic protozoa (e.g. plasmodium, toxoplasma, leishmania and trypanosoma), which lack the ability to synthesize purine nucleotides de novo, it has been suggested that AK inhibitors could attenuate their growth by blocking the purine salvage pathway [5,6].

Until recently, the AK activity and genes were only known to be present in eukaryotic organisms [3]. However, Long et al. [7] have recently reported identification of an enzyme from *Mycobacterium tuberculosis* (Accession no. P83374) that corresponds to the only known bacterial AK. The identification of AK activity in *M. tuberculosis* is of much interest because AK can permit the intracellular conversion of nucleoside analogs to toxic anti-metabolites and thus could prove useful in treating multidrug-resistant tuberculosis. The identification of a protein that carried out AK function in *M. tuberculosis* (referred to here as MTub-AK) was of much importance to us due to our long-standing interest in AK and related enzymes [3]. We have previously carried out extensive studies on the effects of adenosine analogs on mammalian cells, and many different kinds of mutants affected in AK were isolated and characterized [8]. We were also among the first to clone and characterize the AK cDNA as well as its gene structure from mammalian species [2,9,10]. Our biochemical studies on AK have led to the discovery of phosphate or pentavalent ion dependency, a property shared by eukaryotic AKs and also other enzymes of the PfkB family [3,11,12]. In the present work, we have further characterized MTub-AK and compared its properties with mammalian AKs, both in vitro and in a cellular setting. The results of our studies show that MTub-AK behaves very differently from mammalian AKs in various respects and it is unable to metabolize various adenosine analogs in cellular systems. These results raise important concerns regarding the functioning of this protein as AK in cells.

2. Materials and methods

2.1. Reagents

D-[1-^{3}H] ribose (20 Ci/mmol), D-[1-^{3}H] arabinose (20 Ci/mmol), D-[^{14}C] fructose (300 mCi/mmol), D-[1-^{3}H] xylose (15 Ci/mmol), and D-[2,8-^{3}H] adenosine (40 Ci/mmol) were purchased from

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American Radiolabeled Chemicals (St. Louis, MO). Adenosine analogs were from Sigma–Aldrich Co.

2.2. Expression of human, Chinese hamster and MTub-AK in E. coli cells

The full length sequence of MTub-AK (*Rv2202c*, Accession no. P83734) was PCR amplified from *M. tuberculosis* DNA using specific oligonucleotide primers. The AK cDNA for human (Accession no. NM_006721.2) and Chinese hamster (P55262) have been described in earlier work [2,9,13]. These sequences were subcloned into pET15b, and *E. coli* BL21 cells were transformed with these plasmids. To examine the effect of adenosine analogs, *E. coli* cells containing these plasmids were grown overnight at 37 °C in LB medium. Cells transformed with the same plasmid containing no insert were used as control. Next day, the overnight cultures were diluted 1:500 with fresh media in presence of 0.1 mM IPTG to induce the AKs. After 1 h, different concentrations of the adenosine analogs were added. After 4 h, the growth of cells in each culture was determined by measuring their optical density at 600 nm. Assuming the cell growth observed in the absence of any drug as 100%, relative growth in presence of adenosine analogs was determined. For purification of MTub-AK, *E. coli* BL21 cells containing this gene were induced with 0.1 mM IPTG for 6 h at room temperature. The $(His)_6$-tagged protein was purified to >90% purity by nickel affinity and gel-filtration chromatography and stored at −20 °C in 10% glycerol.

2.3. Studies with CHO cells expressing human or MTub-AK

Toy^r-4 is a toyocamycin-resistant mutant of CHO cells, which lacks AK (<0.5% of wild-type) due to a large deletion in the AK gene [14]. We have transfected Toy^r-4 cells with the mammalian expression plasmid pcDNA3.1, harboring either human AK cDNA or MTub-AK DNA. Stable transfectants expressing these genes were obtained by growing the cells in presence of G-418 (650 μg/ml) for more than 1 month. The relative plating efficiency of cells expressing either the human or MTub-AK in presence of various adenosine analogs was determined as described in earlier work [9,15].

2.4. Enzyme activity assays

AK activity was measured using [^{3}H]-adenosine as described previously [11,15]. All assays were carried out at 37 °C in 20 mM Tris–maleate buffer (pH 7.0) containing 10 mM KCl and 10 mM $MgCl_2$. The concentrations of Ado and ATP, when held fixed, were 10 μM and 5 mM, respectively. Assays for human and *E. coli* RK were carried out as previously described [16]. The catalytic efficiency of each enzyme for different substrates was expressed as a function of k_{cat} and K_m (k_{cat}/K_m in min^{-1} mM^{-1}). The k_{cat} was calculated by dividing V_{max} by the molar concentration of the enzyme in each reaction, as described previously [11].

3. Results

3.1. Sequence similarity and phylogenetic analysis of MTub-AK

As mentioned previously, AK belongs to the PfkB family of carbohydrate kinases, which are identified by the presence of two highly conserved sequence motifs in the N- and C-terminal regions [1,3]. Despite the presence of these conserved signatures, proteins of the PfkB family exhibit low overall sequence identity (~20%), which often prevents determination of substrate specificity of a new member. The function of MTub-AK also could not be identified based on its amino acid sequence [7]. In fact, pair-wise sequence identity shows that MTub-AK is more closely related to ribokinases and fructokinases than AK from various sources [3,7]. This inference is also clearly evident from the phylogenetic tree shown in Fig. 1. In this tree, MTub-AK branched as a distantly related member of the RK and fructokinase clade, whereas AKs from different sources formed a distinct clade.

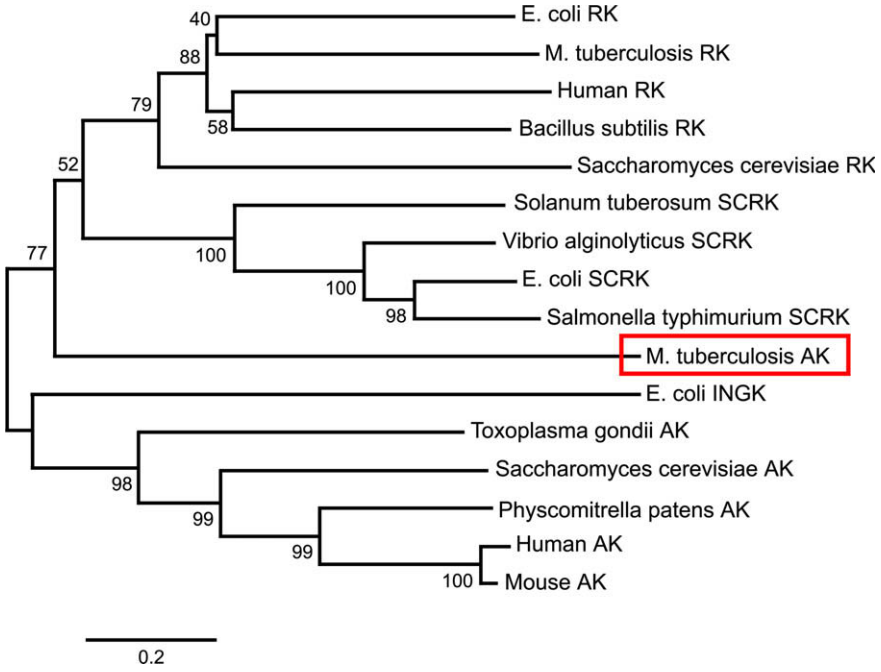

Fig. 1. A consensus neighbor-joining tree for the PfkB family of proteins based on 100 bootstrap samples showing the relationship between MTub-AK and other related proteins. The tree was constructed as described in earlier work [10] and the numbers on the nodes are bootstrap scores. The accession numbers and other information regarding tree construction methods are provided as Supplementary data information. The abbreviations for the enzymes are: SCRK, fructokinase and INGK, inosine-guanosine kinase.

3.2. Effect of expression of M. tuberculosis, human, or CHO-AK on the ability of E. coli cells to metabolize adenosine analogs

Since Long et al. [7] have reported that this MTub protein functions as AK despite exhibiting greater sequence similarity to the RKs and fructokinases, we have tested whether this enzyme can phosphorylate various nucleoside analogs in a cellular setting. To study this, *E. coli* BL21 cells were transformed with pET15b vectors harboring MTub-AK, CHO-AK, or human AK. *E. coli* cells transformed with an empty vector served as a control. Upon addition of IPTG, all of these cells expressed comparable levels of the recombinant proteins as determined by SDS–polyacrylamide gel-electrophoresis (results not shown). We have tested the effects of various adenosine analogs on the growth of these cells. Since most adenosine analogs are prodrugs that need to be phosphorylated by AK before they can exert their cytotoxic effects, only the cells expressing active intracellular AK are expected to show sensitivity to such analogs. The results of these experiments for some of the adenosine analogs tested are presented in Fig. 2.

As seen, the growth of normal *E. coli* cells, or cells transformed with an empty control vector, was not affected in presence of different concentrations of various adenosine analogs. These results support that *E. coli* cells do not contain AK and also indicate that other enzymes in *E. coli* are not able to metabolize these analogs to their toxic forms. In contrast to these cells, the growth of *E. coli* cells transformed with either the human or CHO-AK was inhibited in a dose-dependent manner by very low concentrations of different adenosine analogs (viz. toyocamycin, tubercidin, 8-azaadenosine and 6-methylmercaptopurine riboside (6-MeMPR)) (Fig. 2). These results provide evidence that AK from human and Chinese hamster sources is functioning in *E. coli* as expected to convert (phosphorylate) these analogs to their toxic forms. However, in contrast to these results, cells transformed with the MTub-AK behaved similarly to the control cells, and their growth was not affected in presence of these Ado analogs. These results showed that MTub-AK is not able to metabolize different adenosine analogs that are efficiently metabolized by other well-characterized AKs. Long et al. [7] have reported that MTub-AK is

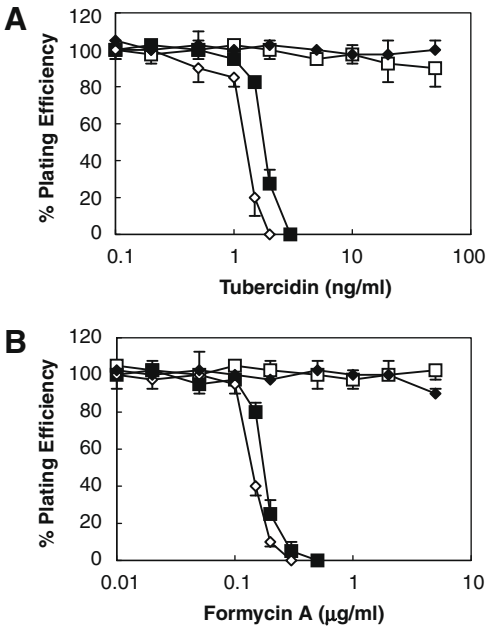

Fig. 3. Effects of adenosine analogs on the viability of wild-type CHO cells (–◇–), Toy^r-4 cells (–♦–), and Toy^r-4 cells transfected with either MTub (–□–) or human (–■–) AK. The plating efficiency of the parental Toy^r-4 cells in the absence of any adenosine analog was regarded as 100%.

responsible for the sensitivity of MTub to 2-methyladenosine. Hence, we also examined the effect of various concentrations (0.1–200 μg/ml) of both 2-methyladenosine as well as 1-methyladenosine on the growth of *E. coli* cells. Surprisingly, both these analogs exhibited no cytotoxicity to various cells expressing different AKs (data not shown). The lack of toxicity of 1-methyladenosine and 2-methyladenosine to cells expressing different AKs indicates that these analogs are either very poor substrates of AK or that their metabolism does not produce toxic effects in *E. coli* cells.

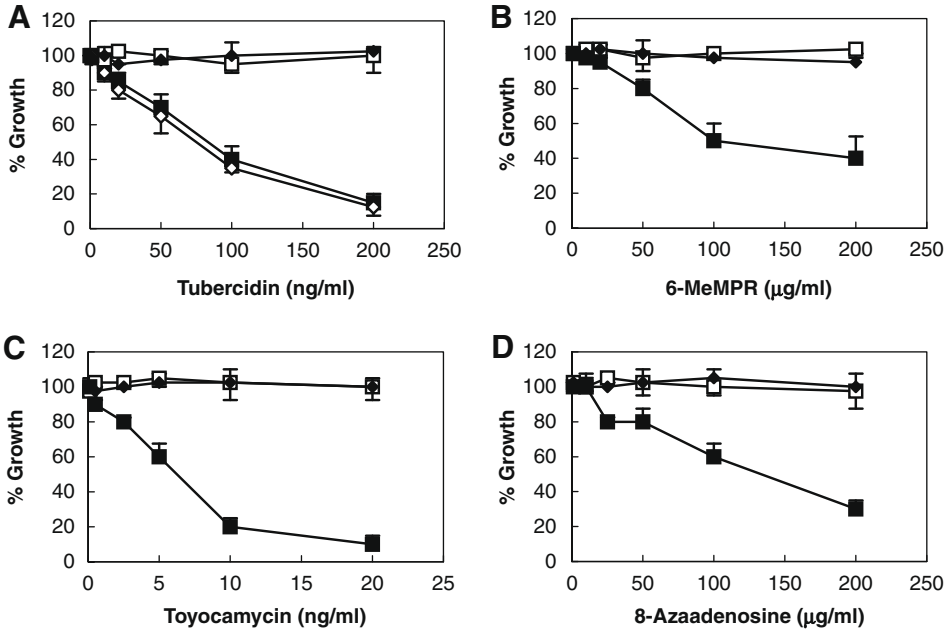

Fig. 2. Effects of adenosine analogs on the viability of *E. coli* cells transformed with plasmids containing MTub (–□–), CHO (–■–), or human (–◇–) AK, or with the vehicle alone (–♦–). The growth of *E. coli* cells transformed with the empty vector in the absence of any drug was regarded as 100%.

3.3. Effect of expression of MTub-AK in AK⁻ mutants of CHO cells

In view of the above results, we have also examined the effect of expression of MTub protein in an AK-deficient mammalian system. These studies were carried out with a CHO cell mutant (Toy^r-4) that is lacking AK [8,14]. We have stably transfected this mutant with the AK cDNA/gene from human (also CHO) or *M. tuberculosis*. The sensitivity of the resulting cell lines to various adenosine analogs was determined by means of plating efficiency experiments. The results of these experiments for two of the adenosine analogs tubercidin and formycin A are presented in Fig. 3. In comparison to the parental cells, the Toy^r-4 mutant is >100-fold resistant to these compounds (Fig. 3) [14]. The Toy^r-4 cells stably transfected with the human AK cDNA (or CHO-AK cDNA, results not shown), became as sensitive to these analogs as the wild-type CHO cells, indicating restoration of AK activity (Fig. 3). In sharp contrast to these results, Toy^r-4 cells transfected with MTub-AK showed no change in their sensitivity to these adenosine analogs and displayed similar levels of resistance as the control Toy^r-4 cells (Fig. 3). Results very similar to those shown were obtained with a number of other analogs (viz. toyocamycin, 8-azaadenosine and 6-MeMPR) that were tested (results not shown). Further, as seen in our studies with *E. coli* cells, 1-methyladenosine and 2-methyladenosine did not exhibit any toxicity towards any of these cell lines up to the highest concentration tested (i.e. 200 μg/ml) (results not shown).

3.4. Biochemical studies on human and M. tuberculosis AK

In view of the differences between the activity of mammalian and MTub-AK in cellular settings, we have also examined biochemical properties of the purified MTub enzyme. One key characteristics of various eukaryotic AKs is their requirement for inorganic phosphate (P_i) for catalytic activity [3,11]. AKs from various sources such as human and Chinese hamster, spinach, yeast and protozoan, are all greatly stimulated by P_i [3,11,15]. In contrast, addition of up to 20 mM P_i had no effect on the activity of MTub-AK [7] (results not shown). In our recent work, we have identified several potent inhibitors of human AK, which inhibit AK in a competitive manner with respect to adenosine [15]. We have tested the effects of these inhibitors on MTub-AK (Fig. 4). Compounds 3998-0118 and 8008-6198, which inhibited human AK in a low micromolar range, produced no significant inhibition of the MTub enzyme up to 1 mM. The other AK inhibitors tested (viz. BTB00475, BTB00480, and BTB00523), whose results are not shown, also gave similar results.

In view of the above differences in the behavior of MTub-AK, we have also measured the catalytic efficiency of MTub-AK for several simple sugars. As seen in Table 1, the MTub-AK showed a ~9-fold preference for Ado over ribose. The activity for fructose was similar to ribose, and other sugars tested (viz. arabinose, xylose, and galactose) showed negligible activity as substrates for this enzyme (results not shown). However, the difference in the behavior of MTub-AK became clearer, when its substrate specificity was compared with some of the other related enzymes such as human AK, human RK, and *E. coli* RK. As shown in Table 1, human AK shows a 820-fold preference for Ado over ribose, whereas human RK and *E. coli* RK favor ribose over Ado by a factor of 141 and 23, respectively. In comparison to these enzymes, the mere 9-fold preference for Ado over ribose seen with MTub-AK indicates that this enzyme is not very stringent in terms of its substrate selection. More importantly, the overall catalytic efficiency of MTub-AK is greatly lower than those of human AK, human RK, and *E. coli* RK. Human AK, for example, is approximately 320-fold more efficient in phosphorylating Ado than MTub-AK (Table 1). It is of particular significance that the catalytic efficiency of human RK and *E. coli* RK for Ado, despite the fact that they showed a preference for ribose over Ado, was higher (*E. coli* RK) or at least comparable (human RK) to that observed for MTub-AK (Table 1). These results indicate that although MTub-AK can phosphorylate Ado in vitro, this activity is very low and it is even less than that observed for *E. coli* RK for Ado.

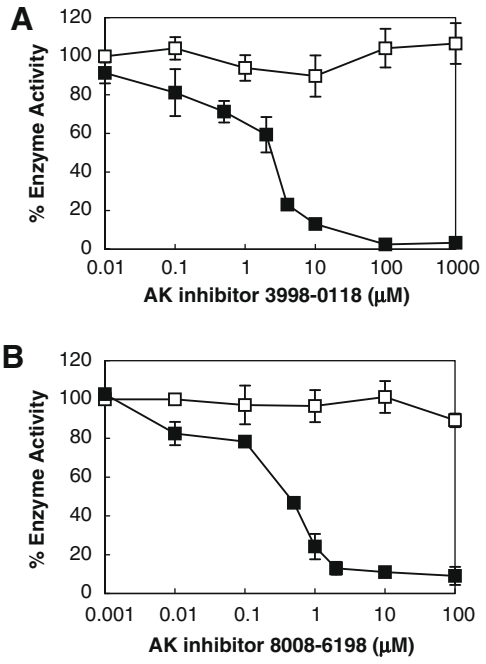

Fig. 4. Effects of two non-nucleoside AK inhibitors (A) compound 3998-0118 and (B) compound 8008-6198 [15] on the activity of purified MTub (–□–) and human (–■–) AK.

Table 1
Substrate specificity of *M. tuberculosis* AK and other related enzymes.

Enzyme	Substrate specificity (k_{cat}/K_m) min^{-1} mM^{-1}		Relative AK activity of various enzymes[a]	Substrate specificity (k_{cat}/K_m) for adenosine/ribose
	Ribose	Adenosine		
MTub AK	1.1	9.9	1	9.9
Human AK	3.9	3200	320	820
Human RK	1100	7.8	0.79	0.007
E. coli RK	12000	530	53	0.044

[a] Assuming the activity of *M. tuberculosis* AK as 1, relative AK activity of other enzymes was determined.

4. Discussion

Members of the PfkB family of proteins, which exhibit AK activity, have only been found in the eukaryotic organisms in the past. Hence, the report of a PfkB family protein in MTub that exhibited AK activity was of much interest, and it necessitated closer examination of its characteristics. This MTub protein exhibited much higher sequence similarity to RKs and fructokinases than to AK from various sources. Further, the functional form of this MTub protein is a dimer [7] similar to RKs from various sources [16,17], whereas all known AKs are monomers [18,19]. Another aspect in which MTub-AK resembles RK more than other AKs is its ionic requirement. Like other RKs, the catalytic activity of MTub-AK is stimulated in the presence of monovalent metal ions such as K^+ [20], while AKs from various sources generally do not require

monovalent ions for their activity [3]. On the other hand, the biological activity of various AK shows a strong dependence upon the presence of pentavalent ions [3,11], which had no effect on the MTub protein.

The results of various experiments presented here further illustrate that the MTub enzyme in question behaves very differently from other well-characterized AKs. Most importantly, this protein, when expressed either in bacterial or mammalian cells, failed to metabolize various Ado analogs to their toxic forms, while the AKs from human or CHO cells were very active in this regard. These results raise serious concerns whether this MTub protein indeed functions as AK in a cellular setting. In this context, it should be noted that although our results confirm that this MTub protein exhibits AK activity in vitro, we find this activity to be very low (about 320-fold lower) in comparison to human AK. This result disagrees with an earlier report by Long and Parker [21], who reported comparable specific activity of both human AK and the MTub enzymes for Ado. We believe that the catalytic activity of human AK in this earlier study was greatly underestimated in comparison to the MTub protein due to a number of reasons. The AK activity from various sources shows a marked dependence upon the presence of inorganic phosphate [11], which was not added to the enzyme reactions, whereas K^+ required for the activation of the *M. tuberculosis* protein was [21]. Also, the assays for human AK were carried out at pH 6, while those for the *M. tuberculosis* protein were at pH 8. The *M. tuberculosis* protein functions optimally at pH 8 [7], whereas the human enzyme at pH 6 only shows half to a third of its maximal activity (pH 7.5–8.5) [22].

Another factor to consider in determining whether this MTub protein functions as AK in cells is the relative abundance of Ado and other simple sugars in cellular environment. Ado exists in micromolar amounts in cells [3], whereas sugars such as ribose and fructose are in millimolar ranges, as reflected in the K_m values of the enzymes metabolizing cellular Ado and sugars [3]. Therefore, although the MTub protein in question shows 9-fold higher catalytic efficiency for Ado over ribose in vitro, which is unusual and it could be due to convergent evolution [7], it is more likely involved in the metabolism of ribose or some other sugar than Ado in a cellular environment where the sugar exists in great excess over Ado. In this context, the inferences from the recently reported crystal structure of the MTub protein [23] should also be interpreted carefully. Although this structure was solved with a bound Ado molecule in the active site, it is clear that ribose can also bind to the same site. Most of the residues (four out of five) that form polar contacts with the ribose moiety of Ado in MTub-AK (Asp12, Gly48, Asn52, and Asp257) are conserved in *E. coli* RK (Asp16, Gly42, Asn46, and Asp255) [18,19], indicating that the same interaction will occur with ribose. Thus *E. coli* RK should also be able to bind Ado, since it shows ~50-fold higher AK activity than MTub-AK. In contrast, the structure around the purine base of Ado in MTub-AK shows no similarity in the residue conservation and arrangement to other known structures of AK [18,19].

As noted earlier, the PfkB family of carbohydrate kinases can phosphorylate the hydroxymethyl group of a wide variety of sugar moieties [1,3]. Recent searches of the Swiss-Prot database with the two conserved sequence motifs found in these proteins (Accession nos. PS00583 and PS00584) identified 213 proteins belonging to this family [3]. These proteins included various known members of the PfkB family of proteins (e.g. RKs, AKs, fructokinase, 1-phosphofructokinase, etc.), but also a number of uncharacterized sugar kinases from bacterial sources. Because the sequence identity among these divergent PfkB members is very low (~20%), the substrate specificity of a new member often cannot be readily predicted. Such was the case for this M. tuberculosis protein, which exhibited greater sequence similarity to RK than to AK, but exhibited very little RK catalytic activity [7]. Although in view of its preference for adenosine over ribose, this protein was suggested to function as AK [7], the results presented here indicate that it seems less likely that this protein carries out adenosine phosphorylation in vivo. It is possible that this protein is involved in the phosphorylation of some other substrate, whose identity remains to be determined.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.06.002.

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